Research Article | Vol. 10, No. 8 | 1 Aug 2019 | BIOMEDICAL OPTICS EXPRESS 4050
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Spectroscopic signature of the pathological processes of carious dentine based on FTIR investigations of the oral biological fluids

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Abstract: The aim of our work is to find a spectroscopic signature of the pathological processes of carious dentine based on the investigations of the molecular composition of the oral biological fluids with the use of FTIR synchrotron techniques. This complex analysis of the obtained data shows that a number of signatures are present only in the spectra of dentine and gingival fluids from the patients developing caries of the deep dentine tissues. The detected features and complex analysis of the quantitative and qualitative data representing signatures of the development of oral cavity pathologies can enhance the quality of dental screening.

1. Introduction

Increased quality of life is a priority trend in national advancement for any developed country. Within the framework of this trend, study of the development of oral cavity diseases caused by cariogenic processes are of great importance due to the direct effect of caries on human health and professional activity [1,2].

The problem of efficient personalized diagnostics of diseases of the deep dentine tissues remains, which is significant and unresolved, as the inflammatory processes in dentine can result not only in the loss of the part of a tooth or even the whole tooth, but also in more serious problems that threaten human health overall [3–5].

The natural reaction of dentine to carious attack, especially at the early stages of pathology development, is the focus of some of the most state-of-the-art investigations [3,5,6]. Currently, these changes can be controlled mainly by a set of rapid analysis techniques based on saliva analysis [7–9] and gingival crevicular fluid [10,11], or inflammatory factors according to serum analysis [12–14]. However, these biological fluids are not in direct contact with dentine, and the changes in their composition can occur due to systemic human diseases, infections and traumas and a result of various stimuli [15–18].

An ideal candidate for the role of a new screening object could be dentine fluid, which plays an important role in the development of dentine caries [19]. Dentine fluid is a derivative of blood plasma, containing serum proteins, immunoglobulins and dissolved mineral substances [20]. Dentine fluid moves from the tooth pulp, fills the branched proliferating dentine channels, circulates inside them and actively interacts with dentine tissue. Bacterial intrusion into the dental canals occurs as a result of compromised dental enamel and cement integrity [21,22]. In this case, bacterial metabolites diffuse through the dental tubules and cause the development of pathological processes in the deep dental tissues [19]. Thus, it is

#369230 https://doi.org/10.1364/BOE.10.004050
Journal © 2019 Received 12 Jun 2019; revised 8 Jul 2019; accepted 10 Jul 2019; published 17 Jul 2019

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highly probable that dentine fluid itself and the markers of pathological processes in the hard dental tissues contained therein can enter the gingival sulcus through the dentine tubules and thus mix with fluid from the sulcus, which is serum transudate [19]. Previous investigations have shown that a characteristic set of proteins and other molecules can be detected in dentine fluid, indicating the development of a pathology, infection or advancement of the inflammatory process in the tissues [4,20,23].

Unfortunately, the use of dentine fluid for diagnosing pathology development in the deep dental tissues in humans is very complicated. The main complexity of such a diagnostic approach is a difficult algorithm involving the extraction of dentine fluid, particularly in the case of fissure caries, when it is necessary to determine if the inflammatory processes occur in dentine. The non-expedient and non-ethical characteristics of this procedure are obvious when considering the beginning of the carious process and the absence of facts confirming inflammation in the tooth dentine.

Extracting gingival crevicular fluid for diagnosing dentine pathology is much simpler and molecular analysis thereof with a selection of markers indicating the development of dentine carious/pathological processes can be performed using molecular identification techniques [10,11,24]. Therefore, it seems reasonable to apply Infrared (IR) spectroscopy as a powerful express analysis technique and informative, precise instrument for studying the molecular and phase composition of biological objects [22]. The identification of prognostic and validation markers for the development of pathological processes is a separate area of interest among the problems resolved using Fourier transform IR (FTIR). IR spectroscopy can be used to determine periodontite levels [11,25] and tendency for caries and to monitor its development [7]. Based on the IR microspectroscopy data, it appears possible to examine the changes in the molecular composition of biological fluids in the oral cavity in pathology development.

The literature does not contain any information on comparison of the molecular composition of dentine and gingival fluids, during pathological changes in dentine to reveal spectroscopic signatures, i.e. markers of pathology.

Therefore, we searched for a spectroscopic signature of the pathological processes of carious dentine based on FTIR investigations of blood, dentine and gingival fluids as well as determined their diagnostic potential for preventive screening of oral cavity pathologies.

2. Materials and methods of investigation

2.1 Experiment design

Ten participants (5 men and 5 women) aged 22–28 years participated in the study. All participants were healthy and did not take antibiotics, medications, smoke or drink alcoholic beverages. All participants did not have any records in their medical report cards for 1 year before the beginning of the experiment. On examination, each participant had teeth with lesion foci related to primary and secondary caries at the stage corresponding to code 1 and 2 according to the International Caries Detection and Assessment System (ICDAS). The participants fasted for 12 hours and did not drink any fluids for at least 2 hours before the sampling of their biological fluids. After preliminary cleaning of the oral cavity, the biological fluids were sampled at 10–12 AM to minimize the effects of the circadian rhythm. Three biological fluid samples were obtained each participant: dentine fluid, gingival sulcus fluid and blood.

2.2 Sampling technique

Taking into account the experience of a number of studies where the capillary effect was used for obtaining microvolumes of fluid from the gingival sulcus, we prepared special tips for our investigations. We sampled the biological fluids using these tips (Figs. 1(a)–1(c)).

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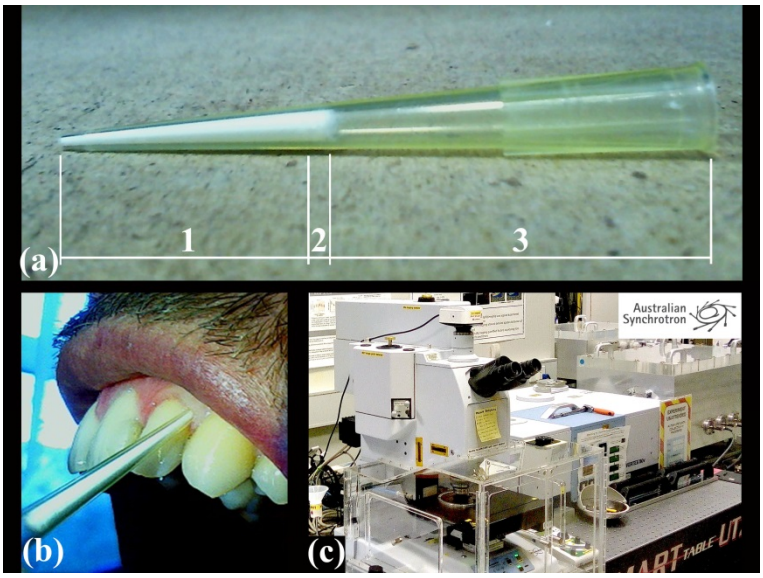

Fig. 1. Microcapillary for sampling the biological fluids. (a) A capillary with the areas filled with (1) pure KBr, (2) nonwoven filter and (3) adapting pipe for microburet. (b) An example of gingival sulcus fluid sampling. (c) Experimental setup for studying the biological fluid samples obtained (HYPERION 3000).

The applied tip represents a microcapillary with an external diameter of 800 μm, and was filled with homogenized potassium bromide (KBr) powder that had been densified using non-woven filter (Fig. 1(a)). The KBr was used as an inert carrier of the investigated fluid, while its choice as a filler was based on the absence of absorption bands within a wide IR spectrum range.

The microcapillary was attached to a sterilized syringe. A difference in pressure in the microcapillary was produced either by the piston tool of the attached syringe or as a result of the use of an evacuating setup. When the required value of the pressure difference was achieved, then the biological fluid entered the KBr.

2.3 Preparation of the samples

2.3.1 Dentine fluid

As indicated above, at examination, each participant had teeth suspicious for enamel surface caries. No obvious signs of periodontitis or gingivitis development were observed.

The development of the carious process in teeth was detected based on our previously engineered approach, where microareas of the hard dental tissues demonstrated a higher fluorescence yield than the areas of intact enamel due to the starting misorientation of the apatite crystals [26].

Participants with caries detected after cofferdam separation of a tooth underwent preparation of both the enamel and dentine using a micro-engine air tip of a spherical doped tungsten–vanadium steel dental drill rotating at 4000 rpm.

After creating a fissure in the masticatory surface in the tooth up to the dentine opening, the infected de-mineralized layer of yellowish dentine was observed. Subsequently, if the examination confirmed the development of dentine caries, dentine fluid was sampled from the prepared cavity using a microcapillary tip and evacuating setup ALP-02. Here, a hermetic seal was made on the masticatory surface of the prepared tooth using the rubber gland, and this construction was attached to the evacuating setup. This allowed us to generate negative pressure under the rubber gland of about 0.9 atm/cm^2 and thus the dentine fluid sample could be obtained in no more than 1 minute.

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2.3.2 Gingival sulcus fluid

Gingival fluid was sampled from each participant from the gingival sulcus of the same tooth from which dentine fluid had been sampled. Here, the participant first thoroughly rinsed their oral cavity preliminarily. Next, to isolate the sampling area, the teeth were edged from the vestibular and oral areas with sterile cotton swabs. The sampling area was dried with air from an oil-less compressor. The gingival sulcus fluid was then sampled using a microcapillary as it shown on the Fig. 1(b).

2.3.3 Blood

Blood was sampled from each participant from the same gingival sulcus following gingival fluid sampling. The gingival sulcus was intubated using a sterile probe and a drop of blood was sampled using a microcapillary.

2.4 Equipment setup and sample scanning

After sampling, the KBr powder from the microcapillaries containing the biological fluids was dried at room temperature, and then examined using IR microspectroscopy (IRM).

The molecular composition of the dentine fluid, gingival fluid and blood was investigated using IR spectroscopy and IRM beamline equipment (Synchrotron, Victoria, Australia), with a Bruker VERTEX 80v spectrometer coupled with a Hyperion 3000 FTIR microscope (Fig. 1(c)) and a liquid nitrogen–cooled narrow-band mercury cadmium telluride (MCT) detector (Bruker Optik GmbH, Ettlingen, Germany) [27]. All synchrotron FTIR spectra were recorded within a spectral range of 3800–700 cm^{-1} at 4-cm^{-1} spectral resolution. Blackman-Harris 3-term apodization, Mertz phase correction and zero-filling factor of 2 were set as default acquisition parameters using the OPUS 7.2 software suite (Bruker Optik GmbH).

For measuring the synchrotron FTIR transmission, small pieces of the powdered sample were transferred and pressed between a pair of diamond microcompression cell windows (Thermo Fisher Scientific, Victoria, Australia), along with a small piece of KBr powder used as an IR background reference [27]. The spectral data were acquired in transmission mode using a 36x objective lens (numerical aperture (NA) = 0.50; Bruker Optik GmbH), a beam focus size of 6.9-μm diameter and eight co-added scans per spectrum. Background spectra were acquired on the KBr, which was well separated from the powdered sample inside the same diamond compression cell, using 32 co-added scans.

Under FTIR, the investigated system is weakly affected by the external impact; therefore, information on the molecular composition of the sample can be obtained without alteration as a result of exposure to irradiation [7,12,14,27].

2.5 Spectral analysis

Spectral data processing, graph plotting, all manipulations of the spectra (removal of the background and correction for atmosphere conditions), averaging of the spectra and data integration, and all calculations, were performed using the OPUS professional software suite (version 7.2, Bruker Optik GmbH). To smooth the spectral data, a second-order Savitzky-Golay filter-polynomial was applied over five data points.

3. Experimental results and discussion

The experimental data obtained by IRM demonstrated that the spectra of the participants' same-type samples comprised absolutely one and the same set of vibration modes. Furthermore, these spectra differed from each other non-significantly only by changes in the vibration band intensity. The spectra of samples averaged over the groups of participants are presented in the Fig. 2, and all other calculations were performed based on the analysis of the averaged spectra. The procedure of averaging for the spectra over the experimental group

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eventually enables the elimination of random experimental errors and the individual features of participants in a particular group [3].

Figure 2 depict the IR spectra of the dentine and gingival fluids and blood. The IR spectra were interpreted based on the data in previous studies that studied samples of biological fluids from the oral cavity, as well as proteins and amino acids, using FTIR [16,17,28–34].

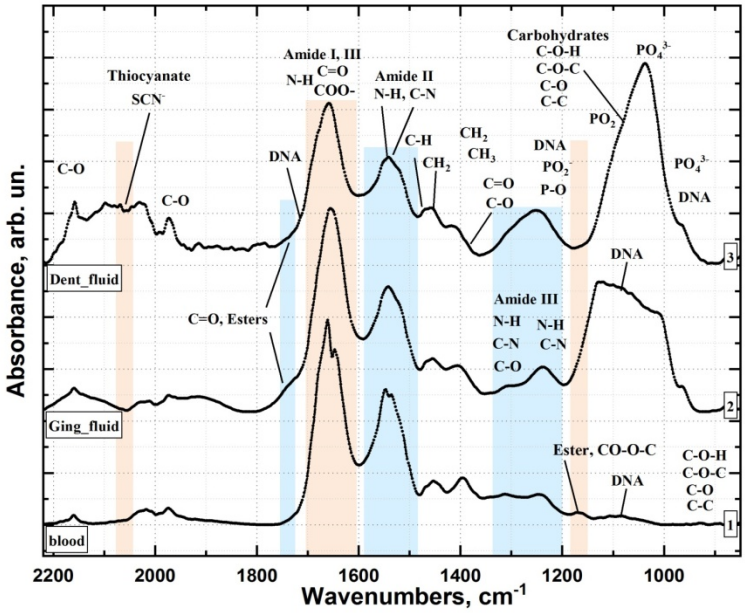

Fig. 2. Comparison of IR spectra in the 2200–850 cm^{-1} range of the gingival and dentine fluids and blood averaged over the participant groups.

The first and most intense group of vibrations, arranged at 1725–1190 cm^{-1}, is attributed to proteins. Bands of secondary Amides could be separated among these groups: Amide I (C = O stretch vibrations in the 1725–1590 cm^{-1} range), Amide II (N–H bend and C–N stretch in the 1590–1500 cm^{-1} range) and Amide III (C–N stretch, N–H bend in the 1350–1190 cm^{-1} range), as well as the vibrations of CH_2/CH_3 groups arranged at 1480–1350 cm^{-1} [25,34–36].

The next large group of vibration bands localized within the 3600 cm–2800 cm^{-1} range is related to the presence of molecular groups attributed to derivatives of the proteins in the samples (α-amylase, albumin, cystatins, mucins) and lipids and fatty acids [7,16,17].

The third group of vibrations in the IR spectra, arranged at 1130–900 cm^{-1}, is attributed to the molecular bonds related to phosphates, glycerophosphates and phospholipids [37,38] and to carbohydrates and derivatives of DNA structures. While this group of vibration bands comprises multiple sets of vibrations related to a mineral component (phosphorus derivatives) for the dentine and gingival fluid samples, the blood sample included low-intensity modes arranged in this spectral interval. These modes are attributed to the molecular groups of carbohydrates and DNA derivatives.

Along with the described main high-intensity groups of modes, more bands were observed in the spectra of the samples, and their intensity was much lower than that of the first three groups. However, their appearance in the spectra is a signature of the proteomics of a specific biological fluid and the development of a pathological process in the oral cavity.

Special attention should be paid to the IR spectra of the three biological fluids in the following spectral intervals: 2200–1800 cm^{-1}, 1765–1725 cm^{-1}, 1171–1160 cm^{-1}, and the vibrations in these regions.

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The first group of vibrations in the 2200–1800 cm^{-1} range was observed only in the spectra of the dentine and gingival fluids. These bands can be attributed to thiocyanates [7,31,32,39], which are indicators of pathological processes in the oral cavity. Their content is increased in caries and periodontal diseases [7]. Despite the high-precision quality of the sampling, we also observed low-intensity vibrations of carbon dioxide (CO_2) absorbed on the gingival and dentine fluids and blood serum in this spectral range. However, the intensity of vibrations in the 2098–2065 cm^{-1} range attributed to thiocyanates observed in the spectra of the dentine and gingival fluids was much higher than the intensity of CO_2 mode.

For the second group of IR vibrations in the 1765–1725 cm^{-1} range, previous data [25,40] show that this spectral band can be attributed to the vibration of the >C = O complex and it can be related to the carboxylic group of an ester (ester carbonyl). The presence of the esters in the hard dental tissue of a human, such as in tooth caries, has been demonstrated previously [25,40]. The authors of these works indicated that esters are more often present in carious tissue than in intact tissue [41].

The third vibration band observed in the 1171–1160 cm^{-1} range in IR spectra is attributed to carbohydrates, and enhancement of their level in oral fluids indicates the development of the carious process, as we have previously demonstrated [7]. While carbohydrates were not detected in the blood sample, their levels in the dentine and gingival fluids were quite high.

4. Analysis and discussion of the obtained results

Based on the FTIR data and an approach tested in our previous works [37,42], we compared the molecular composition of the dentine and gingival fluids and blood. Previously [37,42], we have shown that mathematical estimation of the molecular composition of human biological fluids can be performed based on calculations and analysis of various relationships (coefficients) between the organic and mineral components of the fluid sample. Applying the proposed approach, it is very convenient to use the following coefficients.

The first coefficient, R_1 (Amide II/Amide I), can be calculated from the ratio of the integrated intensity of the Amide II band (CN stretching, NH bending vibrations) in the 1600–1458 cm^{-1} range to that of the Amide I band (C = O stretching vibrations) in the 1720–1600 cm^{-1} range.

The second coefficient, R_2 (Thiocyanate/Protein), proposed previously [39], can be calculated from the ratio of the integrated intensity of the vibration band –N = C = S, arranged at 2100–2050 cm^{-1} and attributed to thiocyanate, to that of the Amide bands (Amide I and Amide II) in the 1720–1485 cm^{-1} range.

The relationship of R_3 (Ester/Amide I) is determined by the ratio of the integrated intensity of the carboxylic group of the complex ester (ester carbonyl) in the 1740–1710 cm^{-1} range to that of the Amide I band (C = O stretching) in the 1720–1600 cm^{-1} range.

These relationships were calculated using OPUS 7.2 (Bruker), and involved a wide set of functional capabilities for processing and estimating the IR spectroscopy data. Figure 3 presents the results of the calculations for coefficients R_1–R_3.

As a reminder, the R_1–R_3 relationships were calculated based on the spectra averaged over the participant groups and on the Fig. 3 is presented mean values of R_1–R_3 relationships. The standard deviation did not exceed 2%.

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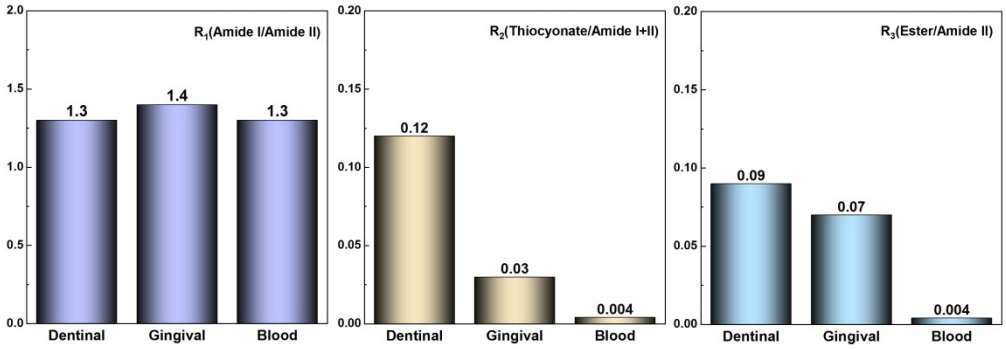

Fig. 3. Values of R_1-R_3 coefficients for gingival and dentine fluids and blood.

In the analysis of the obtained results, under the development of carious dentine pathology, a part of the CN and NH bonds relative to the C = O bonds (coefficient R_1) in the organic component of the gingival and dentine fluids and blood remained unchanged.

However, the most obvious changes in the molecular composition of the biological fluids, namely, the spectroscopic signatures connected with the development of dentine caries, can be detected based on analysis of the R_2 and R_3 coefficients. The minimum and hardly detected values of these coefficients could be revealed in the analysis of blood, while increased thiocyanate (R_2) and complex ester (R_3) levels accompanying caries development [7] were observed in the dentine and gingival fluids (Fig. 3). It is clear that there were higher levels of these markers, related to dentine caries development, in the dentine fluid, but they could also be reliably detected in the gingival fluid. They can indicate that the changes in their composition are related to the development of pathological processes in the deep dentine tissues.

The results we obtained indicate that the composition of the biological fluids represents the development of the pathological processes of dentine caries. The changes determined here, i.e. the composition of dentine fluid are considered reliable spectroscopic signatures of pathologies and can be detected rather easily without the labour-intensive and inappropriate extraction of dentine fluid. This is because these changes are present simultaneously in gingival fluid. Moreover, sampling for screening is not very difficult.

In summarizing the results, we would like to note some important facts. Previous investigations have shown that no integrated marker or the presence of antimicrobial agents in the biological fluids of the oral cavity, on its own has powerful capability for diagnosing the development of dentine caries [43,44]. Therefore, complex analysis of the quantitative and qualitative data representing signatures of the development of oral cavity pathologies can enhance the quality and reliability of dental screening.

5. Conclusion

It is clear that the composition of dentine and gingival fluid is no less complex than that of blood serum. Furthermore, both former fluids are derivatives of blood serum and the majority of molecular groups in all three fluids can be detected in their IR spectra. Nevertheless, our results show that a number of signature modes are present only in the IR spectra of dentine and gingival fluids from patients with developing caries of the deep dentine tissues. This means that both dentine and gingival fluids have high diagnostic potential for the study of pathological processes in the human oral cavity. We observed increased thiocyanates and complex estersin the dentine fluid and gingival crevicular fluid from people with the development of pathological carious processes in the dental tissues.

As its sampling for analysis is not as difficult as that for dentine fluid, the use of gingival fluid for screening would facilitate a shift to personalized medicine and the development of high-technology public health and health care technology as a whole.

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Funding

Russian Science Foundation (RSF) (16-15-00003).

Acknowledgments

The part of this research was undertaken with The Infrared Microspectroscopy (IRM) beamline at the Australian Synchrotron.

Disclosures

The authors declare that there are no conflicts of interest related to this article.

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